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PRINCIPAL INVESTIGATOR: Fangting Wu, Ph.D.

CONTRACTING ORGANIZATION: System Biosciences, LLC
Mountain View, CA 94043

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Fangting Wu

E-Mail: fwu@systembio.com

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14. ABSTRACT
Exosomes are small membrane vesicles secreted by most cell types, functioning as signal transmitters by conveying their bioactive molecules, such as microRNAs and mRNAs to the neighboring cells. It has been reported that normal prostatic epithelial progenitor cells existing in the prostate basal component possess the regenerating ability. However, the connection between prostatic progenitor cells and prostate stem cell is still elusive. The purpose of this study is to determine whether exosomes derived from prostatic epithelial progenitor cells can transfer stemness molecules from prostatic epithelial progenitor cells to prostate cancer cells to contribute prostate cancer stem cell generation and/or maintenance. For this purpose, WPEstem cells were used as a source of prostatic epithelial progenitor cells. We first demonstrate that exosomes derived WPEstem cells can be taken by prostate cancer cells LNCap. Secondly, microRNA profiling, QPCR, and proteomic analysis reveal that WPE-stem cell derived exosomes can deliver certain stem cell signatures such as SOX2 and FOXC2 to LNCap cells. Finally, we found that exosomes derived from WPE-stem cells increase LNCap cells invasion ability. However, we do not observe significant effect of WPE- stem cell derived exosomes on LNCap cell growth, morphology change, sphere formation and CD44+/α2β1 hi/CD133+ population.

15. SUBJECT TERMS
exosomes, prostatic epithelial progenitor cells, prostate cancer stem cells, genetic information transfer

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Introduction

Accumulating evidence indicates that a sub-population of cancer cells with stem-like properties, termed cancer stem cells (CSC); exist in many different kinds of malignancies, which have a pivotal role in tumorigenesis, tumor progression, metastasis and post-treatment relapse. Thus, a better understanding of CSCs, and their derivation and maintenance within tumors is critical to successful cancer therapy. Normal prostatic epithelial progenitors (PEPs) have been shown to exist in the prostate basal component and be capable of regenerating a new prostate gland [1], exhibiting multipotency and self-renewal. However, the connection between PEPs and prostate cancer stem cells (PCSCs) in tumor biology has not been fully addressed. The purpose of this study is to investigate the role of PEPs in generation and maintenance of PCSCs via exosomes. Exosomes are 30-100 nm membrane vesicles secreted by most cell types, and they can function as intercellular transmitters by conveying their contents, such as microRNAs and mRNAs to the neighboring cells. More interestingly, such molecules are biologically functional in the recipient cells [2, 3]. A recent study demonstrated that human skin cancer cells can be reprogrammed into a pluripotent embryonic stem cell-like state by miR-302 [4], suggesting that cancer cells can be switched from differentiated state to poorly differentiated state. Given that PEPs are often home to and incorporate within tumor tissue, we hypothesize that exosomes secreted by PEPs can transfer the bioactive molecules from PEPs to prostate cancer cells (PCCs) to promote their “stemness”. Therefore, the success of this study is expected to provide new insight into basic cancer biology and this knowledge can be used to better target PCSCs as well as develop effective treatments for advanced prostate cancer.

Body

PEP exosomes were transfer to LNCap prostate cancer cells.

To investigate whether PEPex can be taken up by PCCs (Aim 1), WPE-stem cells were used as a source of PEP, and will be called as PEP in this study. We first extracted exosomes from PEP cell culture using traditional ultracentrifugation. PEPex were then validated by western blot using antibody against exosome marker protein CD63 (Fig. 1A) and further confirmed by electron microscope (Fig. 1B).

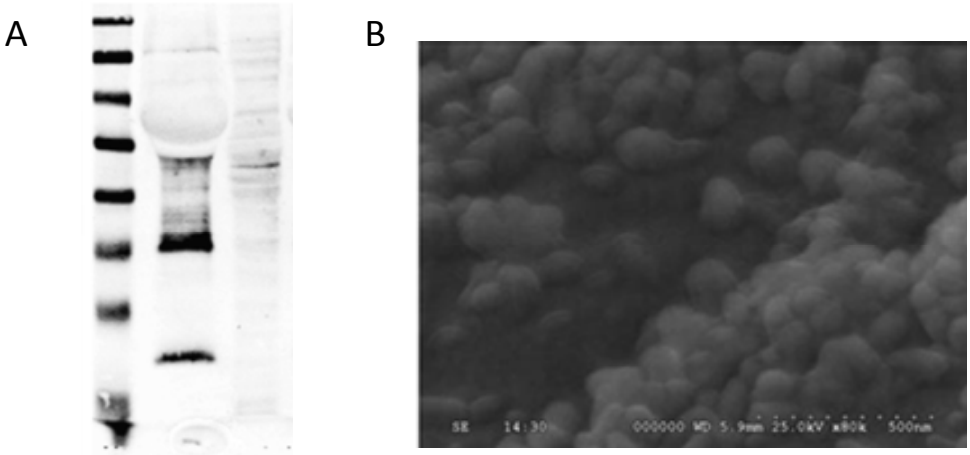

Fig. 1 **Identification and characterization of PEP exosomes.** Exosomes were isolated using ultracentrifugation. (A) Western blot using antibody against exosome marker protein CD63. Exosomes isolated from PEP cell culture and negative control (soluble whole cell lysate) were subjected to western blot and incubate with CD63 antibody. (B) Electron micrographs of WPE-stem exosomes. The image shows small vesicles of approximately 50–80 nm in diameter.

To monitor whether PEPex transfer to prostate cancer cells, we labeled PEPex with a red fluorescent lipid dye (PKH67) that could be made visible with confocal microscopy

(Fig. 2). When purified PKH-labeled PEPex were incubated with LNCap prostate cancer cells [5], LNCAP cells became fluorescent.

Thus, this result suggests that PEPex can be taken up by prostate cells.

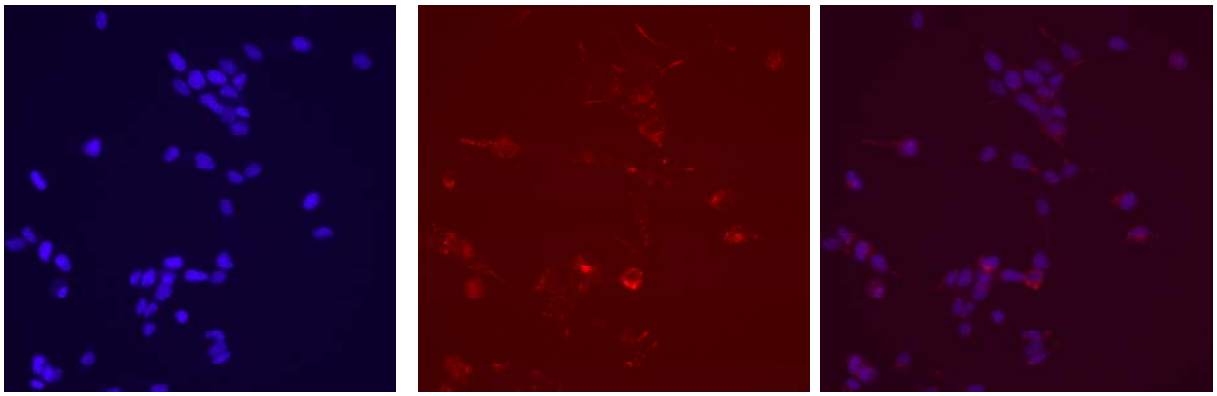

Fig. 2 **Uptake of PEPex by LNCap by fluerescent microscopy.** Exosomes from PEP cells were purified by ultracentrifugation and incubated with PKH26 dye. PKH26 –labeled PEPex were then incubated with LNCap prostate cancer cells. Uptake of PEPex by LNCap cells were monitor by red fluorescence. Hoechst stains nucleus.

PEP exosomes delivers certain PEP signatures to LNCap prostate cancer cells.

To evaluate whether PEPex can transfer PEP signatures to LNCap cells, LNCap cells were incubated with or without PEPex for 1 week up to 2 months. During this period of time, we did not observe any morphological changes in LNCap cells with PEPex (LNCap-PEPex) in comparison of LNCap even after 2 month PEPex incubation.

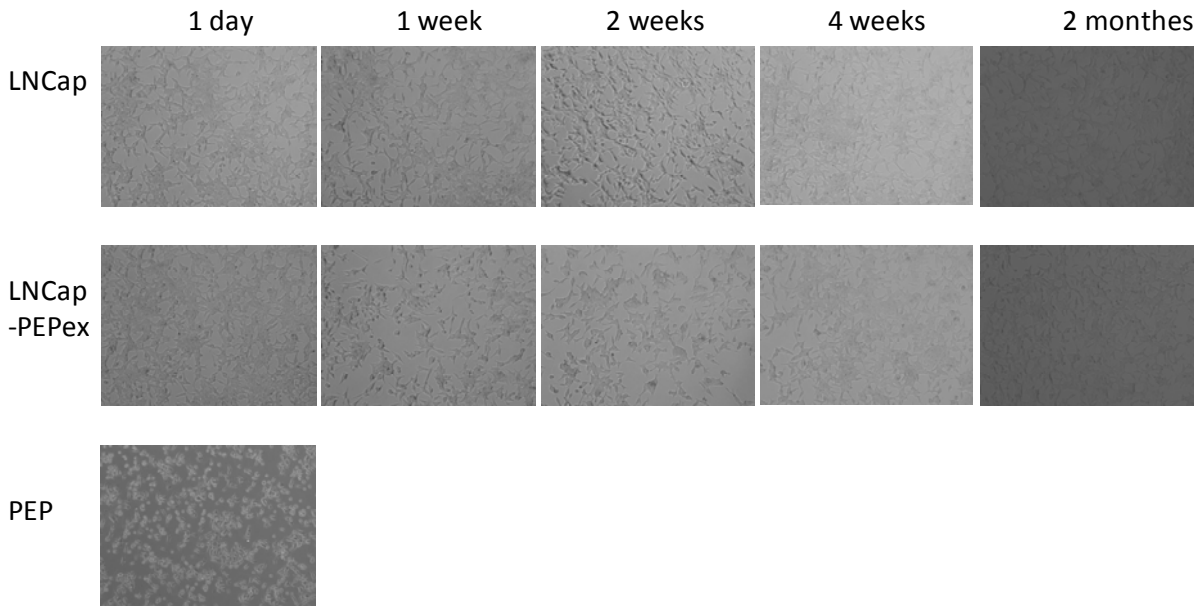

Fig. 3 **PEP exosomes do not change LNCap morphology.** LNCap cells were incubated with or without PEPex for up to 2 months. No morphology change was observed.

It has been shown that Exosomes can transfer mRNAs and microRNA to achieve genetic exchange between cells [2]. We then performed the microRNA profiling in PEP, PEPex, LNCap and LNCAP cells incubated with PEPex for 4 weeks (LNCAP-PEPex). When we compare the expression of 1113 microRNAs between PEP and PEPex, we found 535 microRNAs can be detected in PEP, while 487 microRNAs can be detected in PEPex. When compare the top 50 highly expressed microRNAs in PEP and PEPex , we found 50% microRNAs are overlapped (Fig 4), in line with the previous

finding that bioactive molecules can be packaged into exosomes.

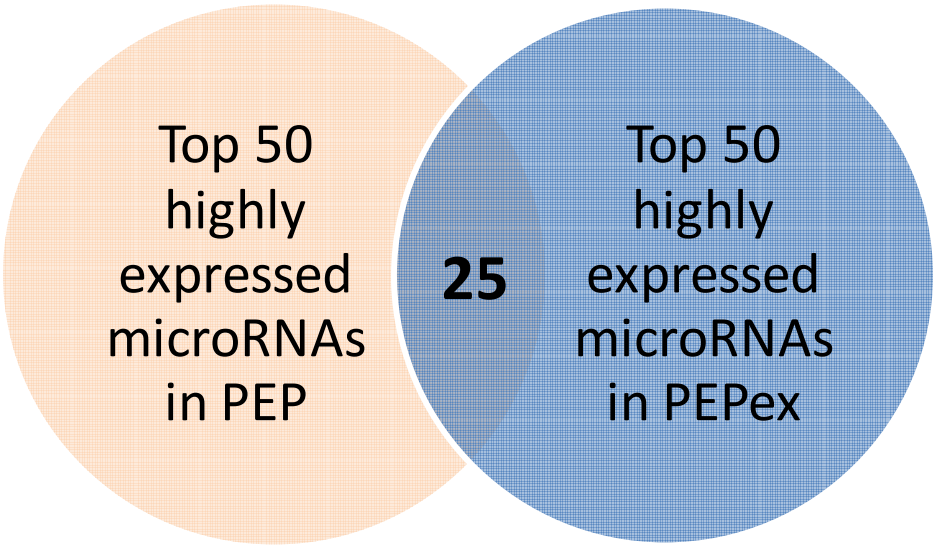

Fig. 4 **Venn Diagram of Top 50 highly expressed microRNAs in PEP and PEPex.** Among top 50 highly expressed microRNAs, 25 microRNAs are shared between PEP and PEPex.

Among these 25 overlapped high expression microRNAs (Table 1),

Table 1. microRNAs with high expression in both PEP and PEPex

hsa-miR-15b	**hsa-miR-23a**	**hsa-miR-30a**	**hsa-miR-222**	**hsa-miR-1260a**
hsa-miR-16	**hsa-miR-23b**	**hsa-miR-30d**	**hsa-miR-378**	**hsa-miR-1274a**
hsa-miR-17	**hsa-miR-24**	**hsa-miR-30e**	**hsa-miR-422a**	**hsa-miR-1274b**
hsa-miR-19b	**hsa-miR-27a**	**hsa-miR-205**	**hsa-miR-720**	**hsa-miR-1290**
hsa-miR-21	**hsa-miR-27b**	**hsa-miR-221**	**hsa-miR-1246**	**hsa-miR-3195**

we noticed that some of them play oncogenic roles and have been reported to play role in maintaining tumor cell self-renewal, promoting invasion and metastasis, resistance to chemotherapy and CSC maintenance e. For example, miR-21 was found to promote ovarian teratocarcinoma PA1 cells by sustaining cancer stem/progenitor populations in vitro [5]. miR- 221/222 was reported to regulate tumor suppressor gene ARHI in prostate cancer [6]. The expression of miR-23a, miR-24 and miR-27a were shown significantly higher in breast cancer with lymph node metastasis [7]. In ovarian ALDH1-

positive cells that associated with chemoresistance, miR-23b, miR-27 and miR-27b were also found overexpressed [8]. miR-378 was shown to function in cellular self-renewal by increasing the expression of SOX2 transcription factor [9]. In CD133+ spheroid-forming subpopulation of OVCAR3 ovarian cancer cell line, miR-205 was found significantly increased [10]. We also noticed that some microRNAs are highly expressed in PEP than PEPex, while some microRNAs are highly expressed in PEPex than PEP. This result is in line with previous notion that some microRNA may be uniquely packed into exosomes [2]. When we compared the microRNA expression in LNCap, PEP, and LNCAP-PEPex cells, we found that around 90 microRNAs display similar expression pattern in LNCap-PEPex and PEP, which express differently in LNCap (Fig 5). For instance, miR-23a and miR-24 expression are high in PEP cells and low in LNCap cells, while the expression of miR-23a is significantly increased in LNCap-PEPex cells. On the contrary, miR-182 expression is low in PEP cell and relative high in LNCap cells, whiles its expression was dramatically decreased in LNCap-PEP cells. This result suggests that certain microRNA signatures do transfer from PEP to LNCap via PEPex.

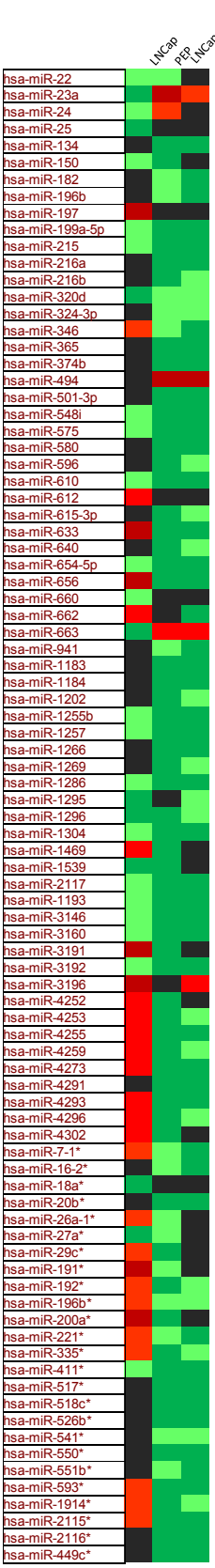

Fig. 5. **Average linkage cluster of LNCap, PEP and LNCap-PEPex.** Data were obtained from microRNA profiling analysis of LNCap, PEP and LNCap-PEPex from week 4. Expression ratios are indicated from low (green), medium (black) to high (red).

To further validate PEP signature transfer to LNCap, we determine the expression of epithelial, mesenchymal and stem cell markers such as Keratin18 (KRT18), E-cadherin (CDH1), N-cadherin (CDH2), Vimentin (VIM), Fibronectin (FN1), Oct3/4, Sox2, Nanog , SSEA, ALDHA, ABCG2, CD44 and AR by real-time PCR (Fig. 6).We found that the expression of FN1, SOX2 and FOXC2 were increase in LNCap-PEPex cells as compared with LNCap cells, while AR expression was reduced. There's no significant change in other epithelial, mesenchymal or cancer stem cell related markers such as VIM, CDH1, Oct4, Nanog, ALDHA, ABCG2, CD44, and CD133 et al. This result suggests that PEPex partially transfers PEP features to LNCAP prostate cancer cells. We also check the targetScan (http://www.targetscan.org/) to search microRNAs that potentially target SOX2, FOXC2 and FN1. Interestingly, we found both SOX2 and FN1 can be targeted by miR-182, and miR-182 expression was found dramatically decreased in LNCap-PEPex in comparison of LNCap cells. Meanwhile, miR-1184 and miR-593 which target FOXC2 were also found decreased in LNCap-PEPex as compared with LNCap (Fig 5). These data suggest that PEPex transfer certain microRNA signatures from PEP to LNCap, which attribute to the changes in certain gene expression including stem cell markers, such as SOX2,

FOXC2.

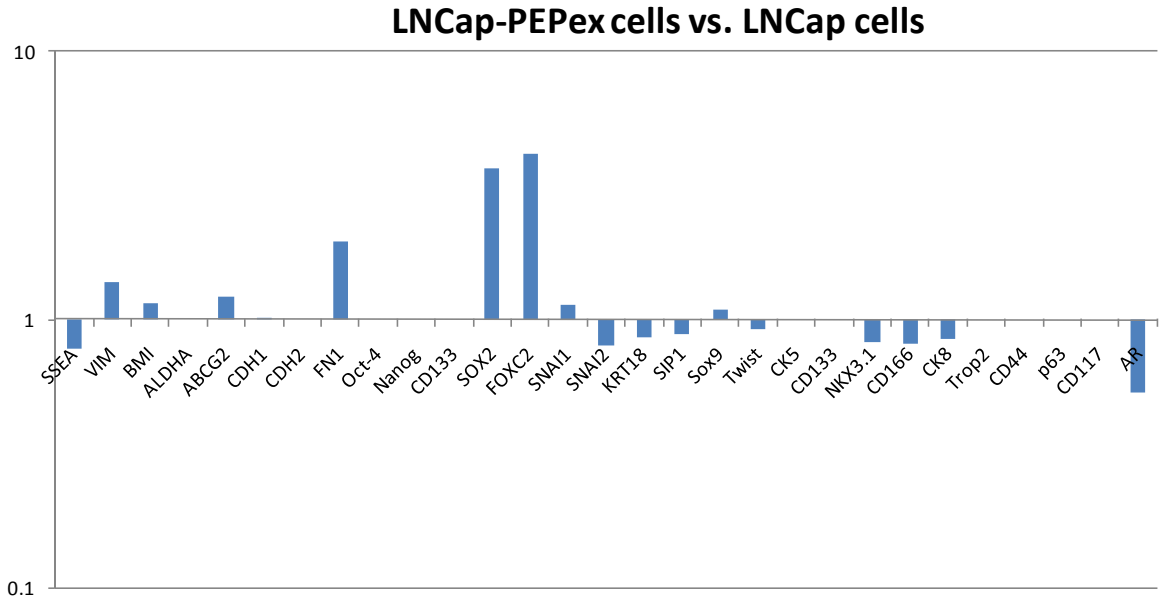

Fig. 6 **Expression of cancer stem cell, mesenchymal and epithelial markers in LNCap, and LNCap-PEPex cells.** RNA were extracted from LNCap and LNCap-PEPex cells and reverse transcribed into cDNA. The expression of Stem cell, mesenchymal and epithelial markers were determined by Real-time PCR.

To further explore the transfer of PEP signature to LNCap through PEPex, we used 2 dimensional fluorescence difference gel electrophoresis (2D DIGE) (Fig. 7) to compare the differences in protein profile between LNCap cells with or without PEPex incubation. Consistent with the microRNA profiling data and mRNA level change, we found that the protein expression in LNCap cells changes after incubation with PEPex with some proteins up-regulated (red spots) and some proteins down-regulated (green spots). The change of protein profile in LNCap cell treated with PEPex may be the direct or indirect results of microRNAs and mRNAs transfer from PEPex. Further studies need

to be done to analyze the identity of these proteins to reveal their functional role.

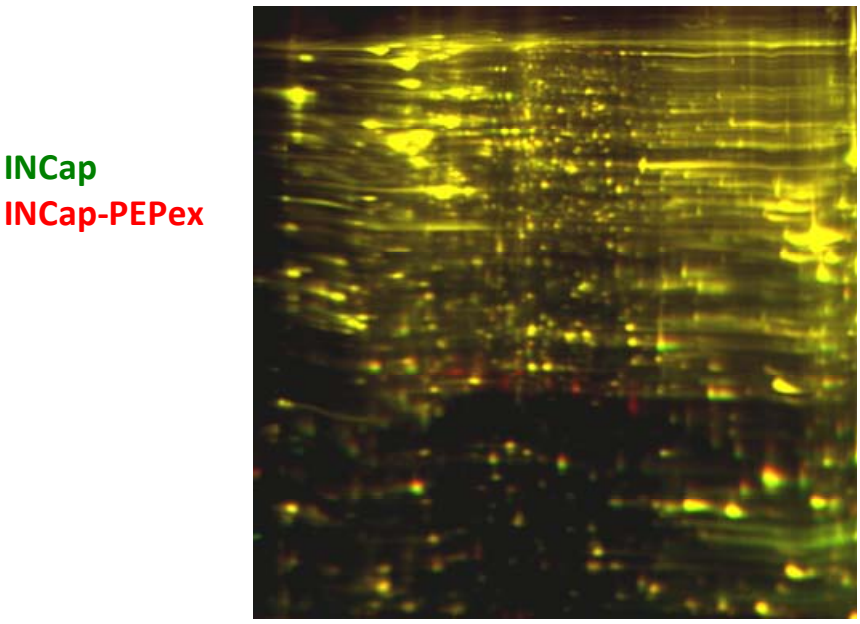

Fig. 7 **Proteomic Analysis of LNCap and LNCap-PEPex cells.** For each sample, 30ug of protein was mixed with 1.0 ul of diluted CyDye. Sample from LNCap cells was labeled with Cy3 (green) and sample from LNCap-PEPex cells was labeled with Cy5 (red), respectively. Gel images were scanned immediately following the SDS-PAGE using Typhoon TRIO.

Taken together, these data suggest that PEP exosomes partially transfer PEP signatures to LNCAP prostate cancer cells, including microRNAs and some stem cell markers.

PEP exosomes increase LNCap cells invasion ability but have no significant effect on cancer stem cell population

After validating bioactive molecule transfer from PEP to LNCap cells through PEPex, the next question we want to address is whether such bioactive molecules would have any effect on cell growth, cell invasion ability or cancer stem cell population.

We first performed the MTT assay to compare the growth rate between LNCap and LNCap-PEPex cells (Fig 8). Both LNCap and LNCap-PEPex display similar growth rate, indicating that PEPex have no effect on cell growth.

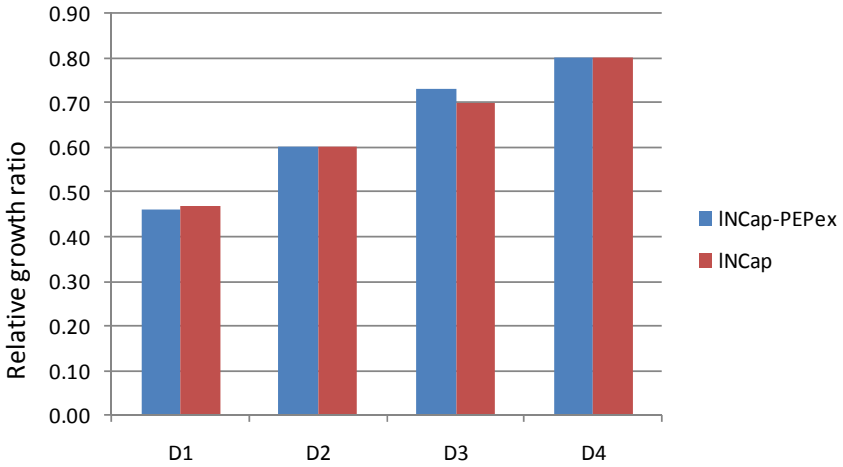

Fig. 8 **PEPex have no effect on LNCap cell growth.** MTT assay was performed to monitor the cell growth rate between LNCap and LNCap-PEPex cells. LNCap and LNCap-PEPex cells show similar growth rate from Day1 to Day4.

Next, we perform the trans-well assay to determine whether PEPex would play a role in cell invasion. Very interesting, we found that LNCAP-PEPex cells are more invasive than LNCAP cells, suggesting that PEPex may promoter cell invasion (Fig. 9). This result consistent with previous finding that FOXC2 and FN1 was up-regulated in LNCAP-PEPex in comparison of LNCAP (Fig. 6), as FOXC2 and FN1 expression link

epithelial-mesenchymal transition (EMT) in different cancers [11-12].

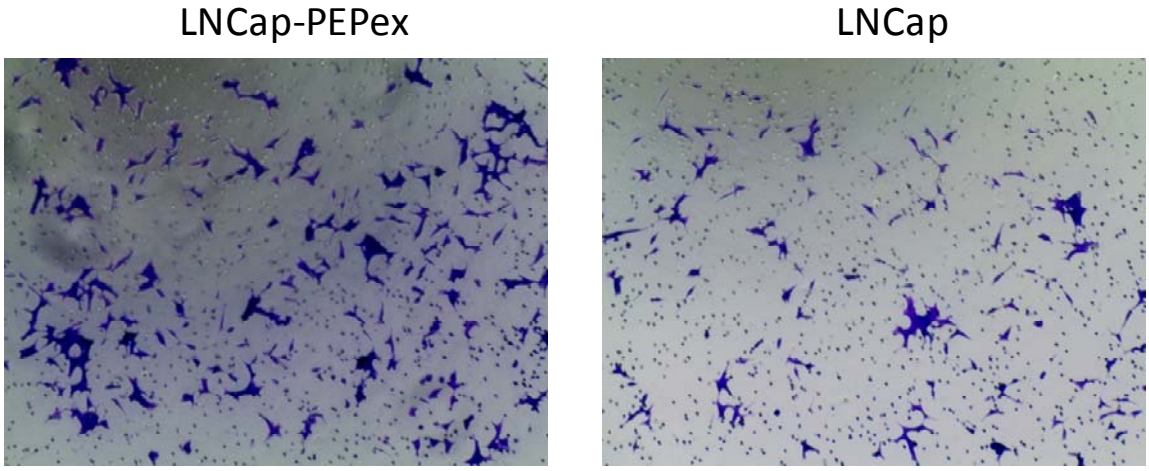

Fig. 9 **PEPex promotes LNCap cell invasionness.**
Trans-well assay was performed to determine the invasion ability between LNCap and LNCap-PEPex cells. Transmembrane cells were stained by crystal violet. LNCap-PEPex cells show higher invasiveness than LNCap cells.

The importance of EMT as a driver of invasion and metastasis is increasingly recognized, and recent evidence has highlighted a link between EMT and the cancer stem cells that initiate and maintain tumors and have also been implicated in invasion and metastasis. Therefore, we further asked whether PEPex would increase cancer stem cell populations in LNCap cells. CSCs have distinct markers and are highly tumorigenic compared to other subsets. Their key features include activation of pluripotency genes (Oct4, Sox2, Nanog), enrichment of population, formation of tumor spheres in low-adherence cultures, and multi-drug resistance. In previous study, we found PEPex have no effect on activation of Oct4 and Nanog, but we did observe increased expression of SOX2 (Fig 6). We then perform the FACS analysis to determine whether incubation with PEPex would increase proportion of cells carrying.

However, we are not able to see any changes in $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ population among LNCap and LNCap-PEPex cells. As SOX2 was shown to increase resistance to androgen depletion in prostate cancer, we further evaluate the sphere formation and UV treatment in LNCap and LNCap-PEPex cells under androgen-deficient condition, we found PEPex have no significant effect on sphere formation as well as cell resistance to UV treatment. Since we do not observe increased $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ subpopuations in LNCap cells after PEPex treatment, this may explain why PEPex have not much effect on sphere formation as well as cell resistance to UV treatment.

Therefore, PEPex have the ability to promote LNCap cells invasion ability, with no obvious evidence showing able to increase cancer stem cell population.

Key Research Accomplishments

- We demonstrated that PEPex can be transferred to LNCap prostate cancer cell.
- We illustrated that PEPex partially delivers PEP signatures to LNCAP prostate cancer cells, including microRNAs, stem cell markers and other proteins.
- Preliminary study suggests that PEPex increases invasion ability of LNCap cell.

Reportable Outcomes

Not yet.

Conclusion

In this study, we have demonstrated LNCap prostate cancer cell is able to uptake exosomes derived from PEP. Though the morphologies of LNCap cells after incubation

with PEPex do not change, microRNA profiling, qPCR as well as proteomic analysis showed that PEPex partially deliver the PEP signatures to LNCap. We observe high percentage (50%) overlap of top 50 highly expressed microRNA in PEP and PEPex, suggesting efficient packaging of small bioactive molecules like microRNAs from PEP to PEP exosomes. As a line of evidence that such molecules do actively transfer from PEP to LNCap cells through PEPex, we found that certain microRNAs which are lowly expressed in LNCap after incubate with PEPex, display increased expression, consistent with the expression level in PEP cells, such as miR-23a and miR-24. Other microRNAs showing the opposite pattern includes miR-182, miR-1184 and miR-593. Most importantly, such transfer of microRNAs from PEP to LNCap thought PEPex has effective biological influence on LNCap cell gene expression as the proteomic profiler between LNCap and LNCap-PEPex shows differences and our qPCR data also showed that certain EMT factors (such as FOXC2 and FN1) and stem cell marker (such as SOX2) are changed. It is worth mentioning that the increased expression of SOX2 and FN1 might due to the decreased expression of miR-182 in LNCap-PEPex as both SOX2 and FN1 are potential targets of miR-182. Similarly, upregulation of FOXC2 in LNCap-PEP may result from reduced expression of miR-1184 and miR-593, as both microRNAs can suppress FOXC2. Given that FOXC2 and FN1 are EMT factors, we further asked whether transfer of PEP signature to LNCap would increase cell invasion ability. In line with the notion, we found LNCap-PEPex cells are more invasive than LNCAP cells. Notably, EMT seems to play a critical role in the generation and maintenance of cancer stem cells, we then further determined whether PEPex would increase cancer stem cell population in LNCap cells. However, we found that PEPex

have no effect on $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ cell population, sphere formation, and cell resistance to UV treatment under androgen-depletion condition, suggesting that PEPex may not be able to increase cancer stem cell population by itself along. This result also explains why we did not observe activation of Oct3/4 and Nanog in LNCAP-PEPex cells.

Future study will be to identify proteins that were differentially expressed in LNCap-PEPex cells in comparison with LNCap, which may reveal new regulators of EMT. In addition, function role of microRNAs that are found highly expressed in PEP and PEPex may worthy further investigation, which will shed new light on exosomes mediated microenvironment regulation.

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Appendices

Acronyms and Symbol Definitions:

CSCs: cancer stem cells

PEPs: prostatic epithelial progentors

PCSCs: prostate cancer stem cells

PCCs: prostate cancer cells

PEPex: PEP-derived exosomes

LNCap-PEPex: LNCAP cells incubated with PEP-derived exosomes

microRNA: miR

CDH2: N-cadherin

VIM: Vimentin

FN1: Fibronectin

KRT18: Keratin18

2D DIGE: 2 dimensional fluorescence difference gel electrophoresis

CDH1: E-cadherin

FACS: Fluorescence-Activated Cell Sorting

EMT: epithelial-mesechymal-transition